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Tuma, J.; Strubbe, J.H.; Mocaer, E.; Koolhaas, J.M.

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S20098 AFFECTS THE FREE-RUNNING RHYTHMS OF BODY TEMPERATURE AND ACTIVITY AND DECREASES LIGHT-INDUCED PHASE DELAYS OF CIRCADIAN RHYTHMS OF THE RAT

Jolanda Tuma,[1] Jan H. Strubbe,[1,*] Elisabeth Mocaër,[2] and Jaap M. Koolhaas[1]

[1]Department of Animal Physiology, University of Groningen, Haren, The Netherlands
[2]Institut de Recherches Internationales Servier, Courbevoie Cedex, France

ABSTRACT

Mammalian endogenous circadian rhythms are entrained to the environmental day-night cycle by light exposure. Melatonin is involved in this entrainment by signaling the day-night information to the endogenous circadian pacemaker. Furthermore, melatonin is known to affect the circadian rhythm of body temperature directly. A striking property of the endogenous melatonin signal is its synthesis pattern, characterized by long-term elevated melatonin levels throughout the night. In the present study, the influence of prolonged treatment with the melatonin agonist S20098 during the activity phase of free-running rats was examined. This was achieved by giving S20098 in the food. The free-running body temperature and activity rhythms were studied. The present study shows that enhancement of the melatonin signal, using S20098, affected the free-running rhythm by gradual phase advances of the start of the activity phase, consequently causing an increase in length of the activity phase. A well-known feature of circadian rhythms is its time-dependent sensitivity for light. Light pulse exposure of an animal

*Corresponding author. Dr. J. H. Strubbe, University of Groningen, Department of Animal Physiology, P. O. Box 14, 9750 AA Haren, The Netherlands. Fax: 31 50 3635205; E-mail: J.H.Strubbe@biol.rug.nl

housed under continuous dark conditions can cause a phase shift of the circadian pacemaker. Therefore, in a second experiment, the influence of melatonin receptor stimulation on the sensitivity of the pacemaker to light was examined by giving the melatonin agonist S20098 in the food during 1 day prior to exposure to a 60-min light pulse of 0, 1.5, 15, or 150 lux given at circadian time (CT) 14. S20098 pretreatment caused a diminished light-pulse-induced phase shift when a light pulse of low light intensity (1.5 lux) was given. S20098 treatment via the food was sufficient to exert chronobiotic activity, and S20098 treatment resulting in prolonged overstimulation of melatonin receptors is able to attenuate the effect of light on the circadian timing system. (*Chronobiology International, 18(5), 781–799, 2001*)

Key Words: Activity; Body temperature; Free-running rhythm; Light pulse; Phase shift; Rat; S20098.

INTRODUCTION

In mammals, clear circadian rhythms have been shown for many behavioral and physiological processes, such as sleep-wake patterns, thermoregulation, cardiovascular function, and many neuroendocrine processes (1). Circadian rhythms in mammals are generated by endogenous oscillators located in the suprachiasmatic nuclei (SCN) of the hypothalamus (1,2). Under natural conditions, these endogenous rhythms are entrained by the environmental light-dark (LD) cycle. In mammals, the SCN receives direct photic information from the environment via neuronal input from the retina (3–5). In addition, light can affect the circadian output generated by the SCN according to a phase-response curve (PRC) (6–8), while under conditions of continuous light or darkness, circadian rhythms free run with an endogenous period that deviates from an exact 24h.

Among the rhythms under SCN control are the synthesis and secretion of the pineal hormone melatonin. From the SCN, a multisynaptic pathway reaches the pinealocytes, in which norepinephrine is released, which activates *N*-acetyltransferase and melatonin production (9). Melatonin synthesis and secretion is elevated during the dark phase, while synthesis is suppressed during the light phase (10,11).

The pineal gland in turn has been shown to play a role in the organization of circadian rhythmicity. There is clear evidence that melatonin feeds back on the SCN, thereby modulating SCN-generated rhythms. In vivo and in vitro studies have shown a phase-dependent inhibitory effect of melatonin on metabolic and neuronal activity in the SCN (12–14). This effect seems to be mediated by melatonin receptors located in the SCN (15,16). Melatonin can affect the circadian output generated by the SCN according to a PRC (17,18). Melatonin administration during the second half of the subjective day causes phase advances, while administration at the beginning of the subjective day causes small phase

delays (8,18,19). Furthermore, a well-studied feature of melatonin is the accelerating reentrainment effect after a phase shift of the environmental light-dark cycle, when an exogenous melatonin injection coincides with the new dark onset time (20). However, removal of the main endogenous melatonin source (pinealectomy) also causes an acceleration of reentrainment after a phase shift of the environmental light-dark cycle (21). Moreover, pinealectomized rodents show disrupted circadian rhythms under continuous light conditions (22). These last findings suggest that melatonin depletion causes enhancement of the influence of the light-dark cycle on the pacemaker system. Therefore, we hypothesized that melatonin receptor overstimulation might attenuate the effects of light on the pacemaker system.

To achieve melatonin receptor stimulation, the melatonin receptor agonist S20098 (Servier, Courbevoie, France) was used. S20098 has specific melatonin agonist properties. It has high specific binding activity for melatonin binding sites (23), and it phase shifts the circadian pacemaker of rodents according to a PRC, as was shown for melatonin (17). Moreover, like melatonin, S20098 accelerates the reentrainment of rhythms of the rat after a phase shift of the light-dark cycle (20).

The majority of studies concerning the role of the pineal gland or melatonin in rhythmicity of behavior and physiology have been performed by removal of the pineal gland (21,22), or by giving short melatonin pulses via daily bolus injections (24–27). Although endogenous melatonin production is characterized by long-term elevated levels throughout the night, little is known about the effect of prolonged stimulation of melatonin receptors throughout the dark phase on rhythmicity. To achieve prolonged and enhanced stimulation of melatonin receptors and to avoid possible entraining effects of daily injections, the melatonin agonist S20098 was added to the food. Since rats are nocturnal animals, most of the food intake and consequent S20098 ingestion took place during the active phase of the rat, corresponding to the phase during which endogenous melatonin is elevated.

In summary, in the present study, the chronobiological consequences of prolonged and enhanced melatonin receptor stimulation by the melatonin agonist S20098 on free-running rhythmicity in rats were examined. Furthermore, the hypothesis that melatonin receptor overstimulation might attenuate the effects of light on the pacemaker system was investigated.

MATERIAL AND METHODS

Plasma Levels S20098

Prior to investigating the influence of prolonged S20098 treatment via the food on circadian rhythmicity, the plasma levels of S20098 were measured. Eight male Wistar rats were individually housed under light-dark 12h:12h (LD 12h: 12h) conditions. The animals had free access to food and water. Under halothane

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anesthesia, the animals were provided with a permanent jugular vein cannula according to the method of Steffens (28), giving the opportunity to take blood samples without disturbing the animals. After surgery, animals were allowed to recover for 2 weeks. After recovery, standard laboratory chow was replaced by food mixed with S20098 in two concentrations, 750 parts per million (ppm) or 1500 ppm. One day after food replacement, the blood-sampling procedure started. Once a day, for a period of 11 days, a 0.6-ml blood sample was collected from each animal. Only one sample per day was taken to prevent a change of fluid and/or food intake because of sampling. Blood samples were taken in a random order at circadian time (CT) 2, 6, 10, 14, 18, or 22 (CT12 is the start of the dark phase). Food intake (g) was measured during 2h prior to taking a blood sample. Plasma samples were obtained by centrifugation for 10 min at 2600*g* and 4°C and stored at −20°C until S20098 was determined using high-performance liquid chromatography (HPLC) with electrochemical detection (Servier, Courbevoie, France).

Free-Running Rhythms in Body Temperature and Activity

We housed 12 male Wistar rats individually in Plexiglas cages ($25 \times 25 \times 37.5$ cm). Food and water were available ad libitum. Under halothane anesthesia, animals were provided with an intraperitoneally implanted transmitter for measuring body temperature and general activity telemetrically (TA10TA-F40, Data Sciences, St. Paul, MN). Signals from the transmitter were picked up by a receiver board underneath the cages, fed to a personal computer, and processed with a specialized recording and analysis system (Dataquest IV, Data Sciences). After surgery, the animals were allowed to recover for 2 weeks. Initially, the animals were housed under LD 12h:12h conditions using dim red light (<1 lux) during the dark phase and bright white light (<100 lux) during the light phase. After 8 days, the LD 12h:12h condition was switched to continuous dim red light (DD; <1 lux). After 16 days in the DD condition, half of the animals received food containing 1500 ppm S20098 ($n = 6$). The other half of the animals ($n = 6$) received control food. Treatment lasted for 13 days, and the animals had continuous free access to the food.

Data sampling of activity and body temperature occurred at 5-min intervals. For the calculation of the free-running rhythm characteristics, τ (period), α (activity phase length), and ρ (resting phase length), the time of the activity, and temperature rise and drop were used as reference phases. The times of the temperature and activity rise and drop were calculated by determining the crossings between a 2h running mean and a 24h running mean of the original data (updated every 5 min). The time points at which the 2h running mean rose above and fell below the 24h running mean were used as the estimates of the start and end, respectively, of the activity or high-temperature phase. Using the start and end

of the activity and high-temperature phases, τ, α, and ρ were determined (see Fig. 1).

Light-Pulse-Induced Phase Delay

Two groups, one of 10 and one of 12 male Wistar rats, were individually housed in Plexiglas cages ($30 \times 40 \times 20$ cm). Food and water were available ad libitum. As in the previous experiment, body temperature and activity were monitored telemetrically from intraperitoneally implanted transmitters. After implantation of the transmitter, the animals were allowed to recover for 1 week under LD 12h:12h. After this recovery period, animals were subjected to constant environmental conditions with light intensity below 0.1 lux to provide free-running rhythms for temperature and activity.

After 14 days of continuous darkness, the standard laboratory chow was replaced by food mixed with 1500 ppm S20098 or control food. One day after the food change, a 60-min light pulse with an intensity of 0, 1.5, 15, or 150 lux was given at the animal-specific CT14. During the light pulse, the animal was placed in its home cage under a light-protected cover containing the lamp that provided the light pulse. After the light pulse was given, the animal was placed in a clean cage, and the S20098-enriched food or control food was replaced by

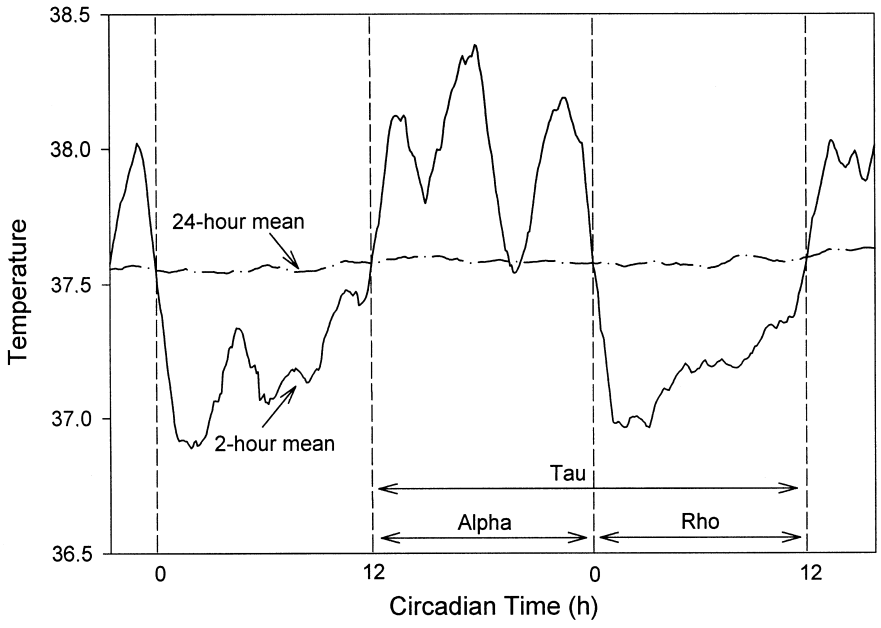

Figure 1. Example of calculation method for τ, α, and ρ based on 2h running mean and 24h running mean.

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standard lab chow. Free-running rhythms were recorded after the light pulse for two successive weeks, after which animals received a new light pulse of different intensity. This procedure was repeated seven times for the first group of 10 animals, and two times for the second group of 12 animals. Each animal received light pulses of different light intensities in a random order. In the first group, light pulses ranging from 1.5 to 150 lux were given; in the second group, light pulses ranging from 0 to 1.5 lux were given. Each rat was exposed to each different combination of S20098 treatment and light pulse intensity only once. An overview of the number of rats exposed to each combination of treatment and light pulse is shown in Table 1.

For calculation of the light-induced phase shifts, the time of the steep rise in temperature at the beginning of the circadian activity period or the time of the sharp drop in temperature was used as the reference phase. For calculation of the time points of steep increase and drop of temperature, see the section on free-running rhythms. Although both temperature and locomotor activity were recorded, only body temperature was used for calculation of the phase shifts because body temperature was more stable and less fragmentary than locomotor activity. Moreover, the locomotor activity rhythm in general corresponds with the body temperature rhythm (29).

The free-running rhythm recorded during the 10 days prior to the light pulse was used to measure the baseline phase. The start of the high-temperature phase on the light pulse day was calculated using linear regression over the baseline period of 10 days and extrapolated to the day of the light pulse (prepulse onset). After the light pulse, 4 days of recording were omitted to exclude possible transients. Linear regression was performed over the next 10 days and extrapolated to the light pulse day (postpulse onset). The difference between calculated prepulse and postpulse onsets was defined as the induced phase shift (Fig. 2).

The animal-specific CT14 was determined in the same way as the prepulse onset.

Only the onsets of the high-temperature phase were used because the onsets were more stable than the offsets of the high-temperature phase.

Table 1. Overview of Rat Exposure

Treatment	Light Intensity (Lux)	Number of Rats
Control	0	6
Control	1.5	12
Control	15	10
Control	150	10
S20098	0	6
S20098	1.5	11
S20098	15	10
S20098	150	10

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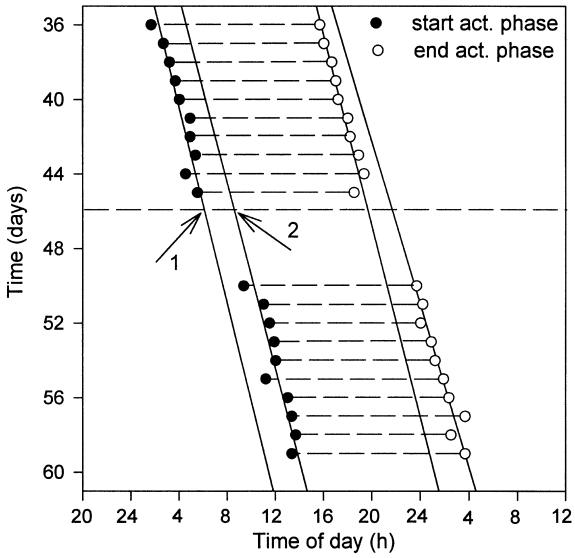

Figure 2. Method for calculating the light-induced phase shift. Start of elevated body temperature phase (closed circles) and end of elevated body temperature phase (open circles) are shown for 10 days prior to light pulse (days 36–45) and for 10 days after the light pulse plus 4 transient days (days 50–59). Arrow 1 indicates extrapolated prepulse elevated temperature phase onset; arrow 2 indicates extrapolated postpulse elevated temperature phase onset. The difference between prepulse and postpulse onset at the day the light pulse was given is the phase shift induced by the light pulse. For a detailed description, see Material and Methods section.

Statistics

Free-Running Rhythms in Body Temperature and Activity

To determine the effect of treatment in time on the start and the end of the activity phase or elevated body temperature phase, a repeated-measures analysis of variance (ANOVA) was performed using the treatment (control or S20098) and time (days) as factors. To determine an effect of treatment on any of the free-running rhythm characteristics, an independent samples *t* test was performed.

Light-Pulse-Induced Phase Shift

To determine the effect of light intensity and treatment, a repeated measures ANOVA was performed with light intensity (0, 1.5, 15, or 150 lux) and treatment (control or S20098) as factors. To determine the effect of treatment per light intensity, a simple factorial ANOVA was performed.

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RESULTS

The 24h profile of plasma levels S20098 in rats having free access to food containing 750 or 1500 ppm S20098 is shown in Fig. 3a, and that for food intake is depicted in Fig. 3b. Food intake was measured over the 2h prior to when the blood sample was taken, which means that the food intake value at CT10, for example, indicates the amount of food eaten between CT8 and CT10. A clear increase in S20098 during the dark phase (CT12–CT0) is shown for both groups,

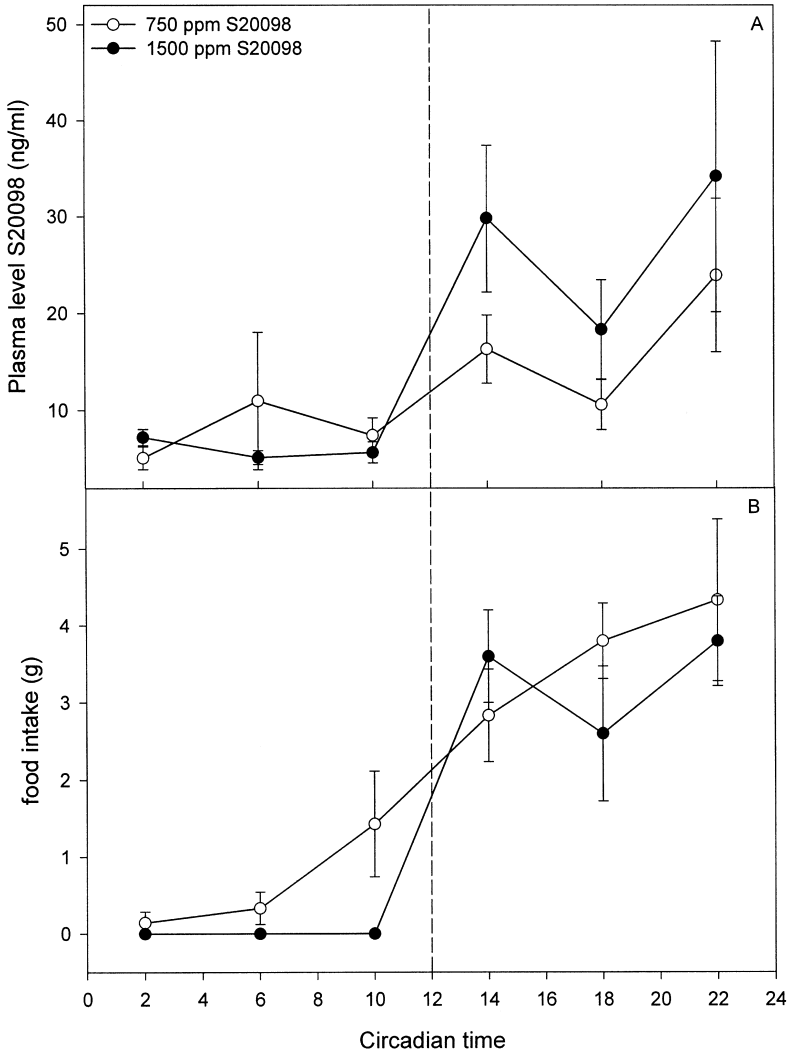

Figure 3. (a) Plasma S20098 levels and (b) food intake throughout the 24h LD cycle during long-term S20098 treatment. Food intake values indicate the amount of food eaten during the 2h prior to the blood sample being taken.

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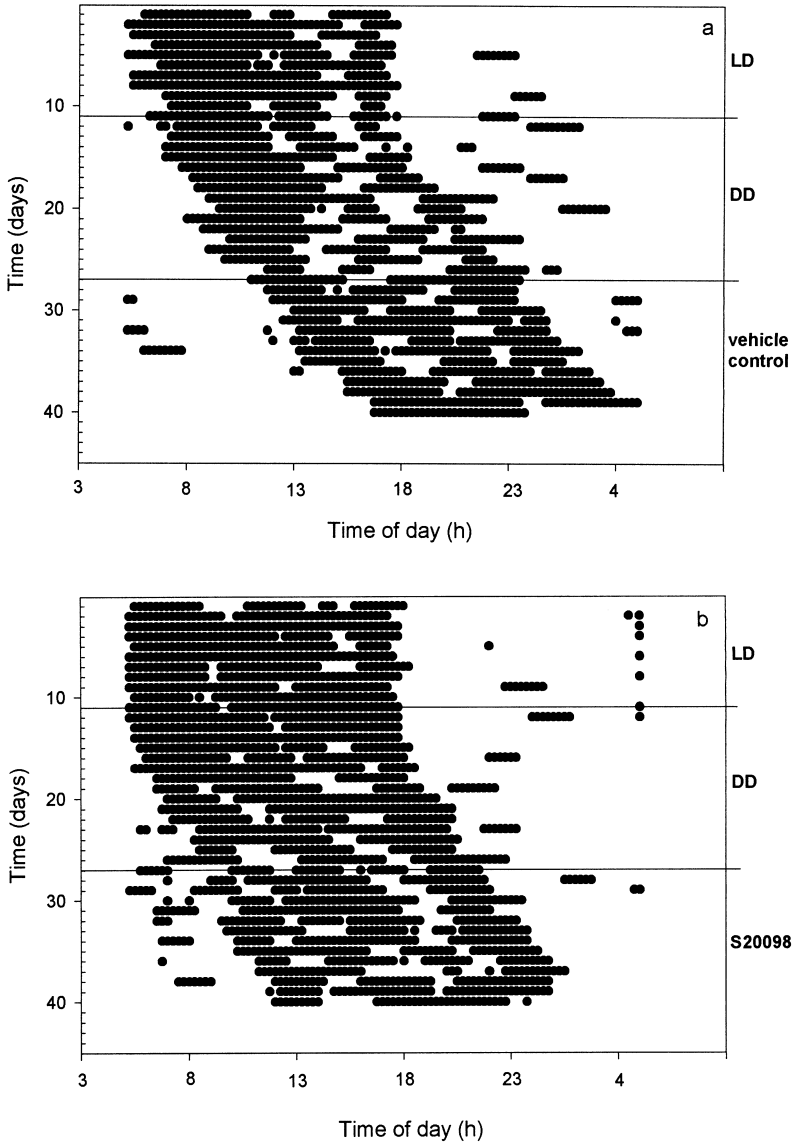

Figure 4. Examples of actograms based on general activity of (a) a control-treated animal and (b) an S20098-treated animal. Days 1–11, LD 12h:12h; days 12–27, continuous darkness; days 28–40, continuous darkness and control or S20098 (1500 ppm) treatment.

while the levels during the light phase are mainly below the detection level (detection level = 5 ng/ml). Especially for the group treated with 1500 ppm S20098, a clear relationship between food intake and plasma levels of S20098 is seen, with peak values for both food intake and plasma levels of S20098 occurring at CT14 and CT22.

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Figure 4 shows two examples of actograms of general activity of one control (Fig. 4a) and one animal treated with S20098 (1500 ppm) animal (Fig. 4b). At day 11, LD 12h:12h conditions were switched to continuous darkness, which was characterized by a clear free-running rhythm. At day 27, standard laboratory chow was replaced by control food or food containing S20098.

Table 2 shows results for τ, α, and ρ based on data collected during the last 7 days of the baseline free run and the last 7 days of the treatment free run. Control-treated animals showed a significant increase in τ (independent samples *t* test, $P = .017$) during the last 7 days of treatment compared to the last 7 days of baseline as measured from the start of the activity phase. Furthermore, control-treated animals showed an increase in the resting phase ρ (independent samples *t* test, $P = .006$) and a decrease in the activity phase α (independent samples *t* test, $P = .046$). Also, as measured at the start of the elevated body temperature phase, control-treated animals show an increase in τ (independent samples *t* test, $P = .003$) and an increase of the low body temperature phase length (independent samples *t* test, $P = .009$), while the elevated body temperature phase length was not changed significantly.

S20098-treated animals showed no change in τ either based on activity or based on temperature data. No significant change was shown for ρ and α in the activity data. However, in the temperature data, a significant increase of the elevated body temperature phase length (independent samples *t* test, $P = .047$) and a trend toward a decrease in the low body temperature phase length (not significant) was shown for S20098-treated animals. The lengthening of the elevated body temperature phase length appears to be due to an advanced start of the elevated body temperature phase in the S20098-treated animals compared to controls (Fig. 5). Repeated measures ANOVA revealed an interaction (Time × Treat-

Table 2. Mean Values ± Standard Error of Mean for τ, α, and ρ Based on Body Temperature and Activity Data

Treatment	Condition	τ Start	τ End	α	ρ
Body temperature					
Control	Baseline	24.299 ± 0.052	24.413 ± 0.045	12.555 ± 0.147	11.744 ± 0.156
S20098	Baseline	24.391 ± 0.019	24.386 ± 0.041	12.307 ± 0.251	12.064 ± 0.249
Control	Treatment	24.607 ± 0.068[a]	24.488 ± 0.106	11.937 ± 0.323	12.551 ± 0.224[a]
S20098	Treatment	24.390 ± 0.032	24.493 ± 0.087	13.081 ± 0.269[a]	11.411 ± 0.331
Activity					
Control	Baseline	24.264 ± 0.037	24.385 ± 0.046	12.647 ± 0.065	11.618 ± 0.069
S20098	Baseline	24.384 ± 0.033	24.447 ± 0.046	12.720 ± 0.283	11.652 ± 0.271
Control	Treatment	24.558 ± 0.106[a]	24.462 ± 0.067	11.937 ± 0.336[a]	12.525 ± 0.282[a]
S20098	Treatment	24.400 ± 0.075	24.507 ± 0.092	13.356 ± 0.323	11.153 ± 0.406

τ was calculated according to both the start and the end of the elevated body temperature or activity phase.

[a]Significant differences ($P < .05$) between control and treatment condition.

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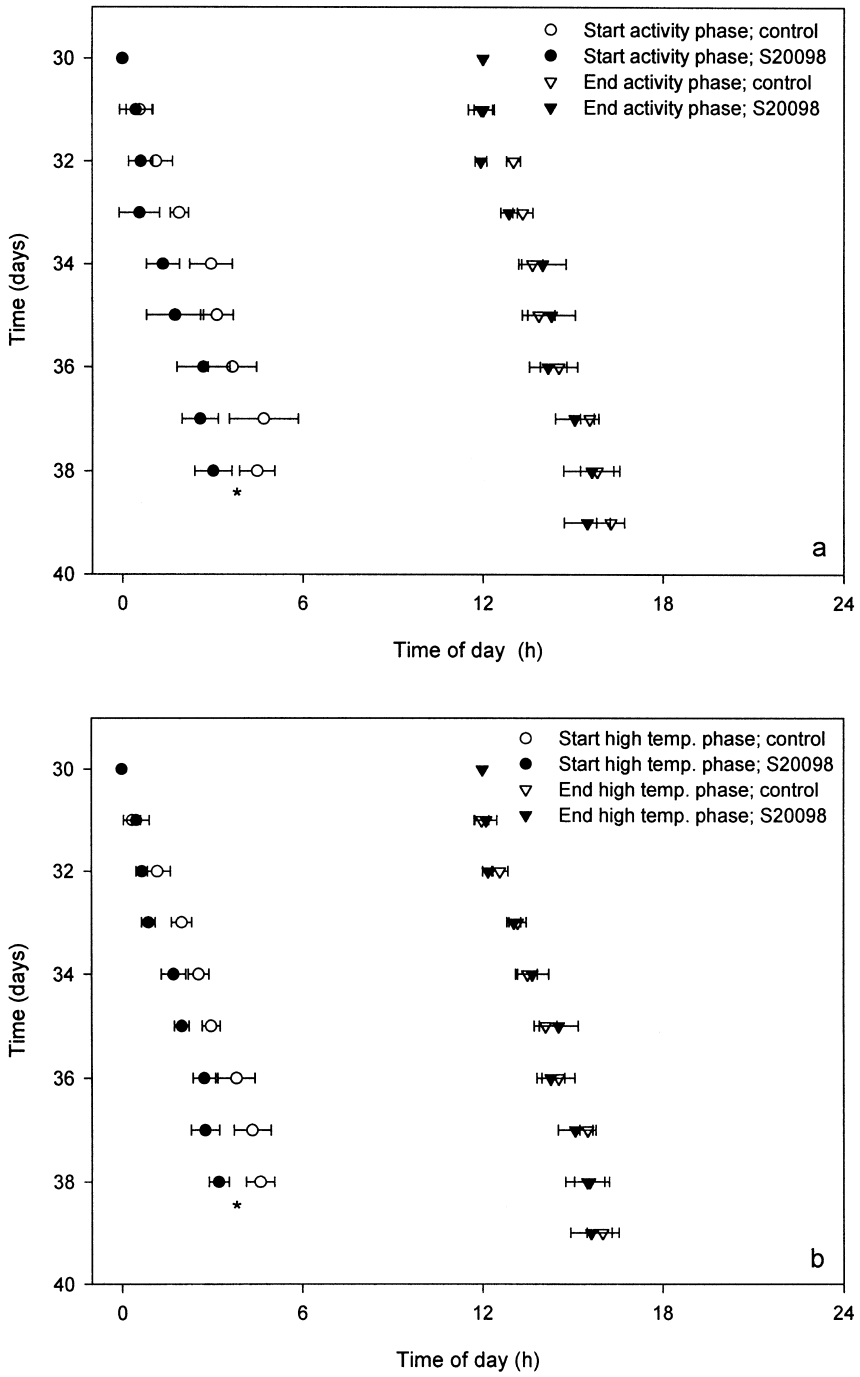

Figure 5. (a) Start and end of daily activity phase. Start of activity phase is shown by open circles for controls and solid circles for S20098-treated animals. End of activity phase is shown by open triangles for controls and by closed triangles for S20098-treated animals. (b) Start and end of daily elevated body temperature phase.

ment) effect both for the start of the activity phase ($F_{8,72} = 2.35$, $P = .026$) and the start of the elevated body temperature phase length ($F_{8,75} = 2.34$, $P = .027$).

Figure 6 shows actograms based on activity and temperature data for a control-treated animal (Figs. 6a, 6b) and an S20098-treated animal (Figs. 6c, 6d) given a light pulse (1.5 lux) at CT14. Figures 6e and 6f show actograms for an animal treated with S20098 (days 10–11) exposed to the light pulse procedure without receiving a light pulse. The actograms based on temperature were achieved by depicting a dot at each 15-min bout when the 2h running mean was higher than the 24h running mean, while no dot was depicted when the 2h running mean was lower than the 24h running mean (see also Fig. 1).

In control-treated animals, a light pulse given at CT14 caused a significant light-intensity-dependent increase in phase delay (repeated measures ANOVA, $F_{3,28} = 7.68$, $P = .001$) (Fig. 7). The size of the phase delay gradually increased with the intensity of the light pulse from about 0 min (0 lux), through 90 min (1.5 lux), 110 min (15 lux), to 135 min (150 lux). Also, in S20098-treated animals, a light pulse given at CT14 caused a significant light-intensity-dependent increase in phase delay (repeated measures ANOVA, $F_{3,26} = 10.07$, $P = .0001$), the phase delay increasing from about 10 min (0 lux), through 45 min (1.5 lux), to 120 min for both 15 and 150 lux.

Although no difference in phase delay was found between control- and S20098-treated animals, using no light pulse (0 lux) or a light pulse of relative high intensity (i.e., 15 or 150 lux), a clear difference was found in the phase delay under the low light intensity of 1.5 lux. For this low light intensity, S20098 significantly attenuated the phase delay from about 90 min in control-treated animals to about 45 min in S20098-treated animals ($F_{1,12} = 5.10$, $P = .036$) (Fig. 7).

DISCUSSION

The present results show that chronic S20098 treatment affected the free-running temperature rhythm of the male Wistar rat by advancing the start of the elevated body temperature phase, while the end of the elevated body temperature phase was not affected. Consequently, the elevated body temperature phase (α) increased, while the low body temperature phase (ρ) decreased.

The treatment method raises the question whether the route of administration was sufficient to exert chronobiotic activity. Therefore, the plasma S20098 levels during S20098 treatment via the food were measured. In comparison to acute oral administration, the plasma levels of S20098, ranging from 11 to 35 ng/ml in the dark phase (Fig. 3) were in the chronobiotic dose-effect range for S20098. That is, acute oral administration in the range of 2.5, 5, and 10 mg/kg resulted in plasma S20098 concentrations of about 20, 62, and 164 ng/ml, respectively (30). The present peak plasma concentrations correspond to acute oral administration with doses between 2.5 and 5 mg/kg. Martinet et al. (30) have

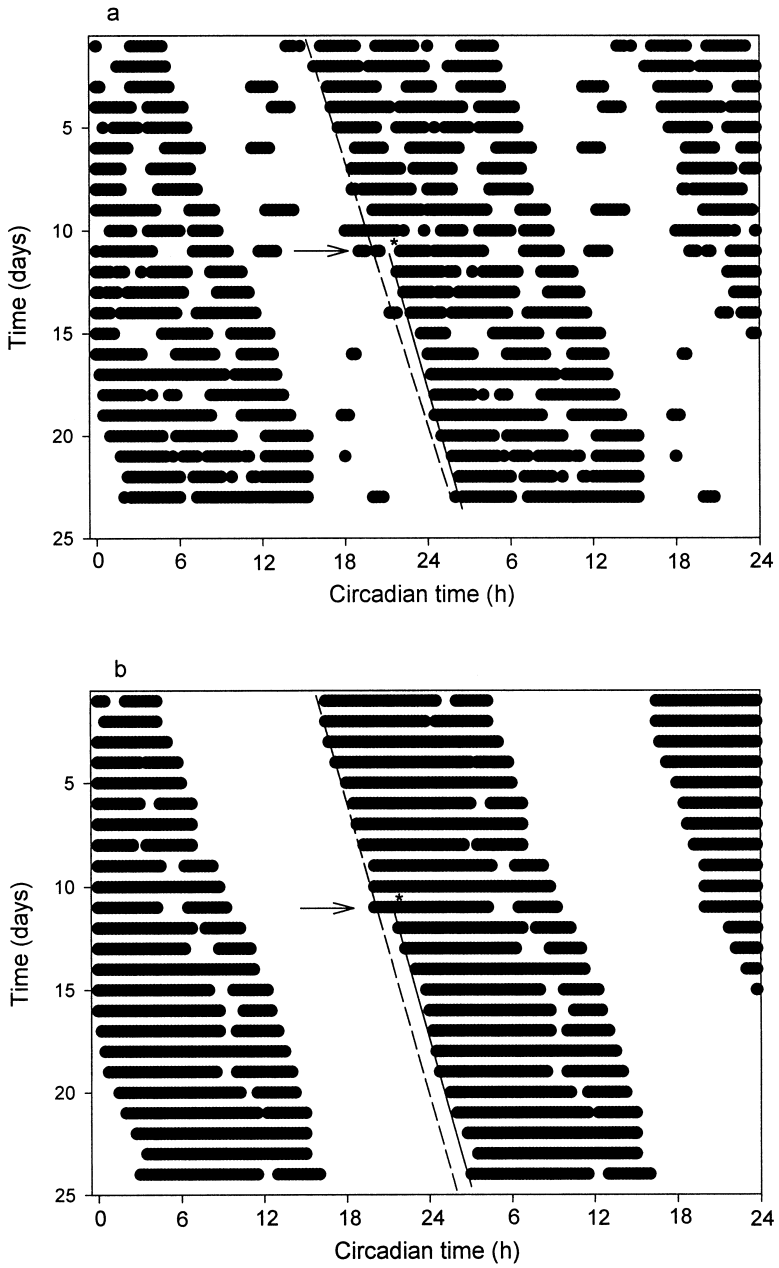

Figure 6. Example of actograms based on activity (a, c, e) and body temperature data (b, d, f) of an animal exposed to a 1.5-lux light pulse and pretreatment with control food (a and b), an animal exposed to a 1.5-lux light pulse and pretreatment with S20098 (c and d), and an animal exposed to a control pulse 0 lux and pretreatment with S20098 (e and f). Arrows and * indicate the day and time, respectively, at which the light pulse was given. Dashed line indicates the prepulse linear regression line; the solid line indicates the postpulse linear regression line.

(*continued*)

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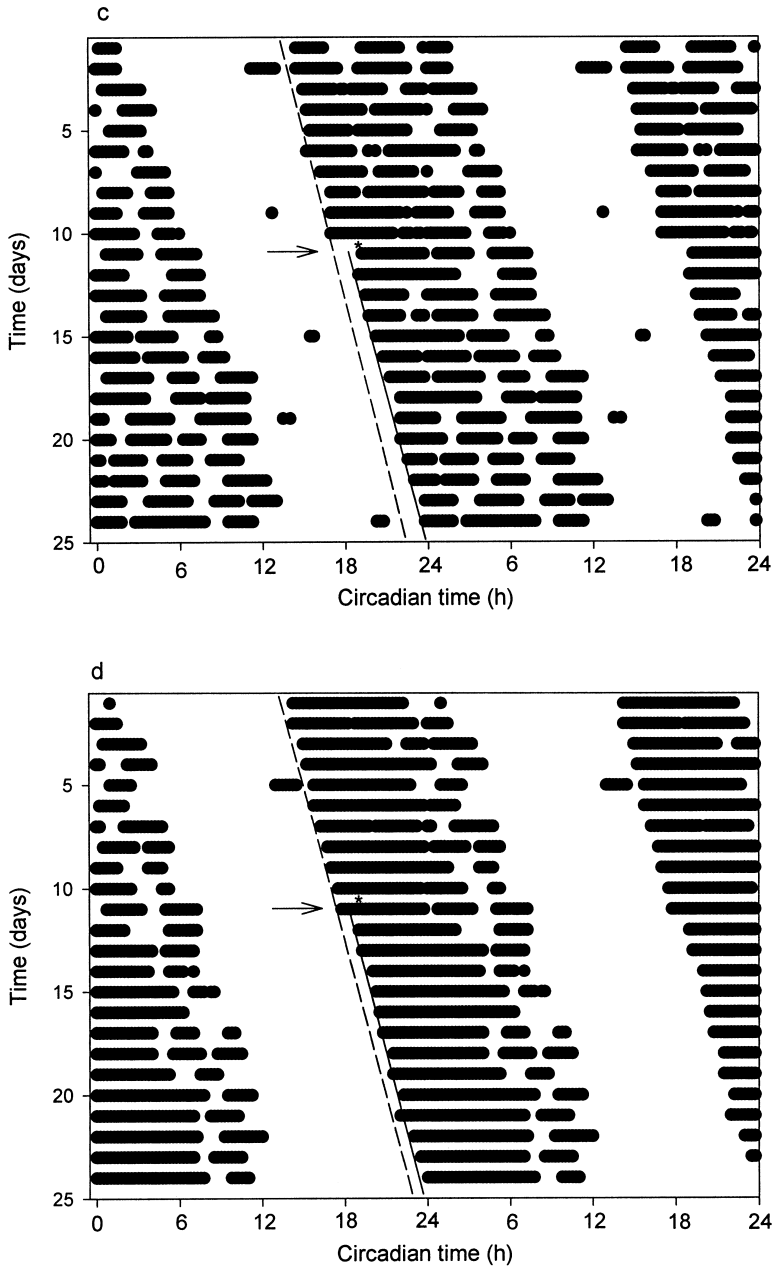

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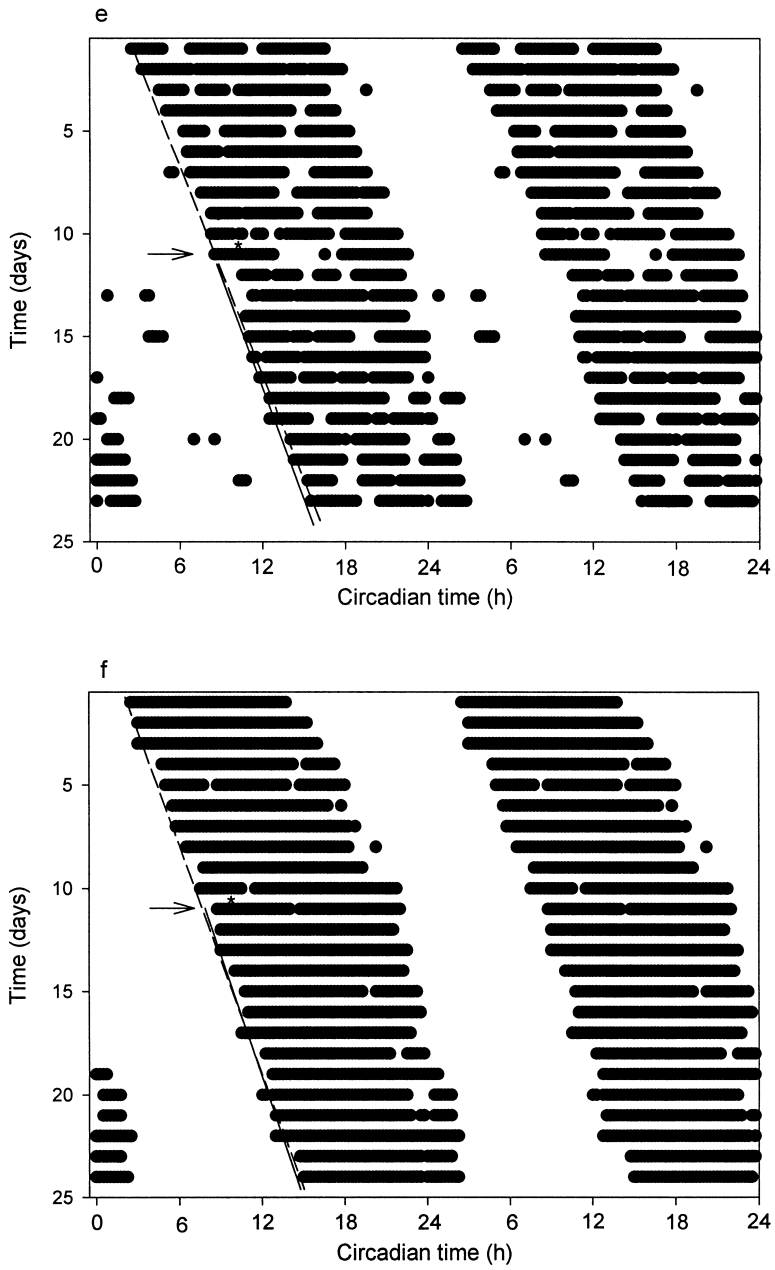

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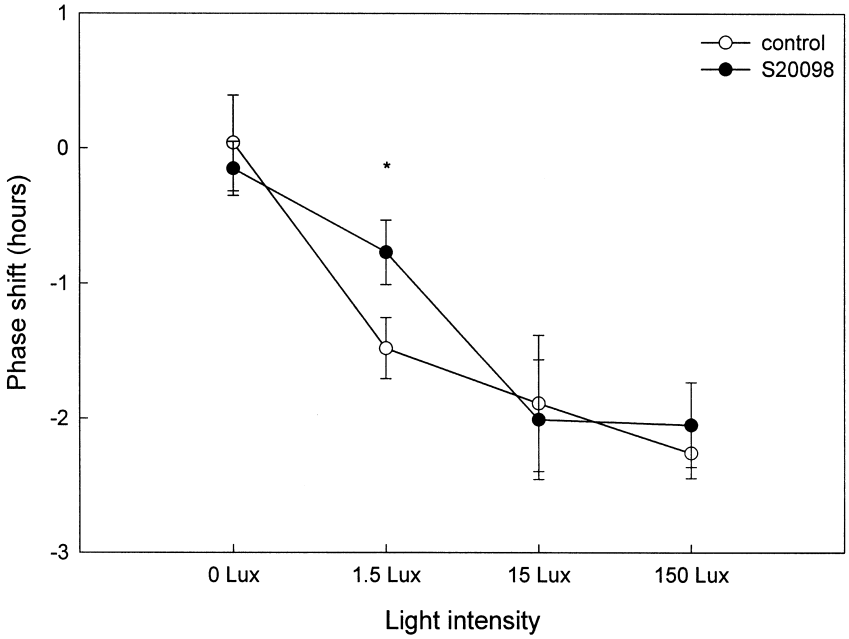

Figure 7. Light-induced phase delays plotted for different light intensities. A 60-min light pulse of 0, 1.5, 15, or 150 lux was given at CT14. S20098-treated animals (solid circles) show a diminished phase delay after exposure to a 1.5-lux light pulse compared to control animals (open circles). *Significant difference ($P < .05$) between S20098 and control.

reported a clear dose-dependent response from 2.5 to 10 mg/kg S20098 (oral administration) with an ED_{50} of 5.7 mg/kg for true entrainment of free-running rhythms in rats (30). Thus, since the nighttime plasma S20098 concentrations were within the dose-effect range for entrainment properties of S20098, the route of administration was sufficient to exert chronobiotic activity.

Indeed, this was shown in the present results by the gradual phase-advancing effect of S20098 of free-running rhythms. Besides an effect on phase, it might be worthwhile to note that long-term S20098 treatment via the food (1500 ppm) did not affect the amplitude of activity (data not shown), but shows a small decrease in nighttime temperature values (about 0.1°C) compared to control-treated animals (data not shown).

Daily melatonin injections have been shown to cause also an increase in α in hamsters housed under entrained short-day conditions (LD 9h:15h) (31). As in the present study, this increase was caused by phase advances of the start of the activity phase, while the end of the activity phase was not affected (31).

In summary, an enhancement of the melatonin signal using S20098 given in the food was sufficient to exert chronobiotic activity. This is shown by the gradual phase advances of the start of the elevated body temperature phase in S20098-treated animals.

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Furthermore, in our second experiment, results showed a light-intensity-dependent increment in phase delay when a 60-min light pulse was presented at CT14. One day of pretreatment with the melatonin agonist S20098 caused a decrease in the light-induced phase delay at low-intensity light pulses (1.5 lux). Partly in accordance with this, Benloucif et al. (32) showed also a reduction in the light-induced phase delay in mice by pretreatment with melatonin. However, they only reported an effect of melatonin when light pulses of high light intensity were presented. Furthermore, in agreement with the present findings is the study performed by Deacon and Arendt (33), in which it was shown that melatonin is able to counter light-induced phase delays in humans.

A possible explanation for the present findings may be that the S20098 treatment phase advances the free-running rhythm acutely; consequently, the light pulse will be given at a time point that does not correspond to CT14. If this is the case, a light pulse given at another time point than CT14 might induce a smaller phase shift. However, this explanation can be excluded since the actogram, showing the free-running rhythm of an animal that received S20098 and no light pulse, did not show any phase advance. Moreover, both control- and S20098-treated groups that received no light did not show a phase shift. In accordance with this, our first experiment showed no acute phase advances, but rather gradual phase advances of the start of the elevated body temperature phase, resulting in a phase advance of about 1 h compared to control-treated animals over a period of 9 days.

In summary, the present results indicate diminished light sensitivity of the pacemaker system, shown by a diminished light-induced phase delay, at least under low light intensity. To strengthen this indication, further research is required to complete a fully phase-response curve for low light intensity light pulses.

In conclusion, S20098 treatment via the food is sufficient to exert chronobiotic activity, and S20098 treatment resulting in prolonged overstimulation of melatonin receptors is able to attenuate the effect of light on the circadian timing system.

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